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(21) International Application Number: PCT/AU87/00070	(74) Agent: SANTER, Vivien; Clement Hack & Co., 601 St. Kilda Road, Melbourne 3004 (AU).
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(71) Applicants *(for all designated States except US)*: BIO-TECHNOLOGY AUSTRALIA PROPRIETARY LIMITED [AU/AU]; 28 Barcoo Street, Roseville, NSW 2069 (AU). MONASH UNIVERSITY [AU/AU]; Wellington Road, Clayton, VIC 3168 (AU). PRINCE HENRY'S HOSPITAL [AU/AU]; St. Kilda Road, Melbourne, VIC 3004 (AU). ST. VINCENT'S INSTITUTE OF MEDICAL RESEARCH [AU/AU]; 41 Victoria Parade, Fitzroy, VIC 3065 (AU).	**Published** *With international search report.*
(72) Inventors; and (75) Inventors/Applicants *(for US only)* : ROBERTSON, David, Mark [AU/AU]; 6 Floreat Court, Glen Waverley, VIC 3150 (AU). McLACHLAN, Robert, Ian [AU/AU]; 106 Male Street, Brighton, VIC 3186 (AU). DE KRETSER, David, Moritz [AU/AU]; 1 Leura Street, Surrey Hills, VIC 3127 (AU).	

(54) Title: METHOD OF ASSAY OF INHIBIN

(57) Abstract

A method of immunoassay for the estimation of inhibin in an inhibin-containing sample which comprises the step of using an antibody directed against inhibin. Preferably, the antibody is contained in an antiserum raised by injecting an animal with an antigen selected from the group consisting of naturally-occuring or recombinant inhibin, or sub-units, fragments or derivatives thereof. The assay may suitably be a radioimmunoassay, a fluorescence-based immunoassay, or an enzyme-linked immunosorbent assay using labelled 58kD or 31kD inhibin as tracer. Tracers and standards for use in the assay are described and claimed.

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METHOD OF ASSAY OF INHIBIN

5   This invention relates to methods for assay of
inhibin, and in particular to methods for immunoassay of
inhibin.

Background Art

          Two forms of inhibin from bovine follicular fluid
10 have been recently purified to homogeneity, with molecular
masses of 58kD and 31kD (International Patent Application
PCT/AU/85/00119 and Robertson et al 1985, 1986). Under
reducing conditions both forms consist of two subunits with
molecular masses of 43kD and 15kD, and 20kD and 15kD
15 respectively. Their primary amino acid structures have been

elucidated following cloning and analysis of cDNA species
derived from bovine granulosa cell mRNA (International Patent
Application PCT/AU86/00097). These studies
indicate that 31kD inhibin is a processed form of the 58kD
5   molecule. 31-32kD inhibin molecules with similar subunit
structures to bFF inhibin have been isolated from porcine
follicular fluid (Miyamoto et al 1985, Ling et al 1985) and
sequenced (Mason et al 1985).

          Currently inhibin activity is measured by a variety
10 of in vivo and in vitro bioassay systems (Baker et al 1981).
These systems are time consuming, expensive, have limited
sensitivity and precision and are of limited practicability in
their application to large sample numbers (Baker et al 1981,
Lee et al 1982).

15 Summary of the Invention

          The present invention relates to more convenient
assays for the estimation of inhibin than have heretofore been
possible. The preferred assays of the invention are
radioimmunoassays and the following description, whilst being
20 directed to the preferred assays, should not be construed as
limiting the invention to radioimmunoassays. Other assays
within the scope of the invention include ELISAs, immunoassays
based on fluorescence detection, and related assays relying on
polyclonal and monoclonal antibodies against inhibin.

25 Preferred Embodiments of the Invention

          According to one aspect of the present invention
there is provided an immunoassay for the estimation of inhibin
in an inhibin-containing sample which comprises the step of
using an antibody directed against inhibin.

30   Preferably the antibody is contained in an antiserum
raised by injecting an animal with an antigen selected from
the group consisting of naturally-occurring or recombinant
inhibin, or sub-units, fragments or derivatives thereof.
Particularly preferred antigens include preparations containing
35 inhibin, purified bovine 58kD inhibin,

purified bovine 31kD inhibin, human inhibin, or human or
bovine inhibin or fragments thereof produced using recombinant
DNA technology.

Suitable animals include mammals such as mice,
rabbits, horses, donkeys, dogs, sheep, and goats, and birds such
as chickens.

Alternatively a monoclonal antibody or an IgG
directed against any of the aforesaid inhibins may be used.

Most preferably the antibody is capable of
neutralizing inhibin bioactivity.

Preferably the immunoassay is further characterized
by the step of using labelled 58kD or 31kD inhibin as tracer.
More preferably said tracer is labelled with ^{125}iodine (^{125}I)
with an enzyme, or with a fluorescent marker.

Preferably the assay is a radioimmunoassay or an
enzyme-liked immunosorbent assay (ELISA), or a fluorescence-
based immunoassay.

15   The invention provides a method for measuring
inhibin in samples such as follicular fluid or serum from
various species (including humans) wherein concentrations of
inhibin in standards are used to derive the concentration of
inhibin in the follicular fluid or serum by competitive
20 binding of ^{125}I labelled inhibin and inhibin from test samples
with bovine 58kD inhibin antiserum, followed by precipitation
and counting of bound ^{125}I labelled inhibin.

The preferred specific radioimmunoassay system for
inhibin of the invention is applicable to bovine and human
25 follicular fluid and serum, and can employ an antiserum
against (purified bovine) 58kD inhibin with iodinated 31kD or
58kD inhibin as tracer.

According to a second aspect of the invention, there
is provided a method for preparation and purification of
30 ^{125}I-labelled inhibin tracer which comprises the steps of
iodination of inhibin using a Chloramine T procedure and
purification of ^{125}I-inhibin by an affinity fractionation
step.

Preferably the affinity fractionation step uses
35 Matrex Red A.

Preferably the purification procedure additionally
comprises a gel filtration step.

According to a third aspect of the invention there is provided an assay standard selected from the group consisting of naturally-occurring or recombinant inhibin, or fragments or derivatives thereof. Preferably the standard displays parallelism in the assay with the samples under test.

Particularly preferred standards include bovine 31kD inhibin, and partially purified or purified human inhibin.

The conditions of the assay, in particular incubation times, may be varied in order to attain desired levels of sensitivity.

A form of the radioimmunoassay modified for increased sensitivity comprises:

incubating sample and antiserum for 4 days at 4°C, followed by the addition of ^{125}I-31kD inhibin tracer, incubating for 3 days at 4°C and then adding second antibody, precipitating, and counting bound ^{125}I labelled inhibin.

According to one particularly preferred embodiment, suitable for measurement of inhibin in human serum samples, the tracer is ^{125}I-31kD inhibin, and incubation with tracer is performed at elevated temperature (30°C) in the presence of inhibin-free serum, in order to minimize non-specific effects. Suitable sources of inhibin-free serum include steers or other castrated male animals, oophorectomized women, women with premature ovarian failure, and post-menopausal women.

Brief Description of the Drawings

Figure 1 shows the fractionation of ^{125}I-58kD and ^{125}I-31kD inhibin on analytical SDS-PAGE under reducing conditions.

Figure 2 shows the time course of immunization of a rabbit with 58kD inhibin.

Figure 3 shows the in vitro neutralization of bFF inhibin by an antiserum raised to 58kD inhibin.

Figure 4 shows the radioimmunoassay dose response curves of bFF, hFF, purified 58kD and 31kD inhibin and bovine granulosa cell culture medium (BGCM) using either ^{125}I-31kD or ^{125}I-58kD inhibin as tracers.

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Figure 5 shows the profile of inhibin _in vitro_ bioactivity and immunoactivity following fractionation of bFF through the various steps of the inhibin purification procedure of Robertson et al (1986).

5   Figure 6 shows non-reduced SDS-PAGE profiles of ^{125}I-58kD and ^{125}I-31kD inhibin following incubation with bFF and serum under conditions used in the RIA of serum inhibin.

Figure 7 shows the effect of temperature on the binding of ^{125}I-31kD inhibin to the antiserum.

10   Figure 8 shows logit-log dose response lines of bovine and human serum, in the plasma RIA system employing ^{125}I-31kD inhibin as tracer.

Figure 9 shows the ovulation induction regime and serum levels of FSH, LH, inhibin and oestradiol (E_2) in

15 twenty-six women involved in an In Vitro Fertilisation (IVF) programme and one normal woman (FL#27).

Figure 10 is a comparison of plasma E_2 and inhibin levels plotted for some of the data in Figure 9.

Figure 11 shows the correlation between the number

20 of ova produced and E_2 or inhibin levels in serum.

Figure 12 shows the correlation between the numbers of ovarian follicles detected ultrasonically and peak inhibin levels in serum.

Figure 13 shows inhibin, FSH, progesterone and

25 oestradiol concentrations in the sera of non-pregnant subjects (n = 16) on the days following oocyte retrieval.

Figure 14 shows inhibin, FSH, progesterone and oestradiol concentrations in the sera of pregnant subjects (n. = 3) on the days following oocyte retrieval.

30   Figure 15 shows the relationship between serum inhibin and FSH during the luteal phase of non-pregnant subjects. For this analysis, non-detectable inhibin values (n = 29) were assigned the limit of assay sensitivity.

Figure 16 shows inhibin, FSH, LH, oestradiol and progesterone concentrations in the sera of normal women during the menstrual cycle, assayed using anti-31kD inhibin.

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Detailed Description of the Invention

Abbreviations

bFF	:	bovine follicular fluid
hFF	:	human follicular fluid
oFF	:	ovine follicular fluid
oRTF	:	ovine rete testis fluid
HPLC	:	high performance liquid chromatography
SDS-PAGE:		sodium dodecyl sulphate
		polyacrylamide gel electrophoresis
RIA	:	radioimmunoassay
SS	:	steer serum
BS	:	bull serum
CS	:	cow serum
PMS	:	human post-menopausal serum
HFP	:	human female plasma

Preparations

(a)   Purification of bFF inhibin

The purification of bFF 31kD and 58kD inhibin was based on the procedures described previously (Robertson _et al_ 1985, 1986)(Figure 5):

(a)   bFF was fractionated on a Sephacryl S200 (9 x 90 cm) gel filtration column in 0.05M ammonium acetate pH 7.0.

(b)   The void volume fraction from (a) was precipitated at pH 4.75 and fractionated on Sephadex G100 (9 x 90 cm) in 4M acetic acid.

(c) and (d)

Peak I (58kD inhibin) and Peak II (31kD inhibin) fractions from (b) were fractioned on an RPSC Ultrapore column (0.46 x 7.6 cm, Beckman) using a 0-50% acetonitrile gradient in 0.1% trifluoracetic acid. In Figure 5 the continuous line indicates optical density at 280 nm (a) and (b) and 254 nm (c) and (d).

Hatched area denotes inhibin bioactivity.

o---o RIA with ^{125}I-58kD inhibin as tracer.

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o___o RIA with ^{125}I-31kD inhibin as tracer.

Vo = void volume.

BSA = bovine serum albumin (mol. wt 67,000).

OVA = ovalbumin (mol. wt 43,000).

5   The purified inhibin was stored in SDS electroelution buffer (approx. 3% SDS in 10 mM NH_4HCO_3) prior to iodination. For bioassay, samples were methanol precipitated at $-20°C$ in order to remove SDS and solubilized by heating at $37°C$ for 1 hour and sonication.

10   Similar profiles of both bio- and immunoactive inhibin were observed at each stage of the inhibin purification procedure. The biological to immunological activity ratios for a number of purified 31kD and 58kD inhibin preparations using both tracers in the radioimmunoassay ranged

15 from 0.30 - 0.43.

(b)   Sample Preparation

Human follicular fluid was obtained at oocyte collection in the in vitro fertilisation programme at the Queen Victoria Medical Centre/Epworth Hospital, Melbourne. It

20 was charcoal treated (100 mg/ml dextran-coated charcoal for 1 hour at $4°C$), lyophilised, stored at $-20°C$ and resolubilized prior to assay by sonication in assay buffer or culture medium. Ovine follicular fluid (oFF) was obtained by aspiration of ovaries collected at a local abattoir. Ovine

25 rete testis fluid (oRTF) is a lyophilised inhibin preparation (Baker et al 1985). Rat ovarian extract was a charcoal-treated rat ovarian cytosol preparation. Details of the biopotencies of these inhibin preparations are outlined in Table 1.

30   Blood was collected in lithium heparin tubes from 40 women undergoing ovulation induction therapy (clomiphene citrate and human menopausal gonadotrophin treatment) with plasma estradiol levels at time of plasma collection ranging from 40-2,900 pg/ml. Equal aliquots of serum from each

35 subject were combined to produce a plasma pool designated as

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Human Female Plasma pool (HFP). Plasma from four
post-menopausal women aged 52 and over were combined to give a
pool designated Post-Menopausal Serum pool (PMS).

5   Bovine blood, ovaries and testes were collected on
ice from a local abattoir and processed within one hour. All
samples were stored at $-20°C$ after snap freezing in solid
CO_2/ethanol. Blood pools from adult intact (BS, n = 9) and
castrate (SS, n = 1) male, and female (CS, n = 10) cattle were
allowed to clot overnight at $4°C$ prior to centrifugation and
10 storage. Bovine ovarian follicles were hemisected and
granulosa cells were collected by aspiration and cultured for
40 hours at a concentration of 10^5 viable cells per well
(Costar 48 well plate) in 400 μl DMEM/Fl2 complete medium.
Media were stored frozen at $-20°C$ prior to assay. Testes from
15 four bulls were decapsulated and homogenised in equal w/v
Dulbecco's phosphate buffer using an Ultra-Turrax tissue
disperser (Janke and Kunkal KS, Staufen FRG) and centrifuged
at 100,000g x 1 hour at $4°C$ and stored at $-20°C$. Prior to
assay the supernatants were charcoal treated with an equal
20 volume of 1% Norit A in Dulbecco's phosphate buffer and
incubated at $4°C$ for 30 minutes prior to centrifugation and
bioassay (Au et al 1983).

         Immunization Procedure
         Purified 58kD inhibin (14 μg in 500 μl Dulbecco's
25 phosphate buffer) was emulsified in an equal volume of
adjuvant (Marcol 52 [Esso, Australia]: Montanide 888
[S.E.P.P.I.C., Paris] in the ratio 9:1) and injected into an
intact male New Zealand white rabbit 4 intramuscular and one
subcutaneous sites. Two booster injections of 14 μg under the
30 same conditions were given at six weeks and one year. Serum
was collected throughout for assessment of its in vitro
neutralization activity, its ability to bind iodinated inhibin
and for plasma FSH estimations.

         A similar procedure was used to raise antibody
directed against purified 31kD inhibin.

Analytical SDS Polyacrylamide Gel Electrophoresis

Sera and bFF were incubated at various temperatures
in an equal volume of 100 mM phosphate buffer pH 7.4
containing 0.15M NaCl, 0.1% Triton X-100 and either 0.5% BSA
5 for studies with ^{125}I-31kD inhibin or 0.5% Polypep for
^{125}I-58kD inhibin. Equal volumes (5µl) of sample and 10% SDS
and Dulbecco's Phosphate buffer pH 7.4 (30 µl) were placed in
a boiling water bath for 2.0 minutes then in ice. Ten
microlitres of bromophenol blue (0.006%) in glycerol (62.5% in
10 H_2O) was then added and the mixture centrifuged prior to
electrophoresis on 12.5% slab gels (3 hours, 20-30 mA).
Protein molecular weight markers were either
co-electrophoresed with the iodinated sample in the absence of
bFF and serum or on a separate track in their presence. The
15 gels were fixed and stained overnight in ethanol: H_2O
formaldehyde (180: 420: 100) containing 0.1% Coomassie
Brilliant Blue. Each track was divided into 50 2mm slices and
counted in a gamma counter.

Reversed-phase HPLC

20 ^{125}I-Inhibin was applied to an Ultrapore RPSC column
(0.46 x 7.5 cm, Beckman, Berkeley, Ca., USA) and fractionated
using a 30 min linear gradient of 0.50% acetonitrile in 0.1%
trifluoroacetic acid at 1 ml/min and 0.5 ml fractions using
Waters HPLC apparatus (model 6000A pumps and a model 660
25 Programmer, Milford, Mass., USA).

In Vitro Bioassay

Inhibin activity was determined using an _in vitro_
bioassay based upon the dose-dependent suppression of FSH cell
content in rat pituitary cell cultures utilizing a parallel
30 line bioassay design (Scott _et al_ 1980). The charcoal-treated
bovine follicular fluid preparation employed a lymph reference
preparation with an arbitrary unitage of 1 unit/mg (Scott _et
al_ 1980).

Hormone Assays

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(i)   Rabbit FSH Radioimmunoassay

Rabbit FSH was determined using an RIA kit kindly
provided by Dr. A.F. Parlow (Torrance, Ca, USA)
employing 15% polyethylene glycol to separate bound
5   and free hormone. The sensitivity of the assay was
0.9 ng/ml using rabbit FSH AFP.538.C as standard.
The within assay coefficient of variation was 8.1%
and all samples were assayed in the one assay.

(ii)   Rat FSH Radioimmunoassay

10   Rat FSH generated by the pituitary cells in culture
was measured by a specific radioimmunoassay using
reagents supplied by the NIAMDD. ^{125}I-rat FSH (I_5)
was used as tracer and FSH RP-2 used as standard.
The within-assay coefficient of variation was 7%.

15   (iii) RIA of human hormones

Serum FSH was measured by RIA (Amerlex-M, Amersham,
USA) using 2nd IRP FSH as standard with an
interassay CV of 7.0% from 31 assays. LH was
measured by RIA (LH RIA, Diagnostic Products Corp.,
20   L.A., USA) using the 2nd IRP LH as standard with an
interassay CV of 10.1% from 31 assays. Both
oestradiol and progesterone were measured using RIA
(Coat-a-Count, Diagnostic Products Corp., L.A.) with
interassay CVs of 8.7% and 8.1% respectively from
25   150 assays. Serum beta subunit of hCG was measured
by RIA (B-hCG RIA-Quant, Mallinckrodt Inc. St.
Louis, USA) using the hCG 2nd IS as standard with an
interassay CV of 10.4% from 30 assays.

Calculations

30   The RIA dose-response curves were linearised using a
logit-log dose transformation. Parallelism was assessed from
a comparison of slope values of dose-response curves using the
multiple range test for groups of unequal size (Kramer, 1956)
or by paired t-test. Potency estimates were determined using
35   standard parallel line bioassay statistics. In situations
where non-parallelism was observed between dose response lines

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of unknown and standard preparations, potency estimates were
determined from the ratio of their ED_{50} values. The
sensitivity (ED_{10}) was defined as the mass of hormone required
to give 10% displacement in the assay whilst ED_{50} corresponded
5   to the mass required for 50% displacement.

The index of precision (Gaddum (1933); Finney
(1964)) was used to describe assay precision. The between
assay variation was calculated from the coefficient of
variation of the repeated measurement of a partially purified
10   inhibin preparation. The dissociation constant (K_{dis}) was
determined by Scatchard analysis using ^{125}I-hormone and
increasing amounts of unlabelled hormone. The mass of
^{125}I-hormone used in the analysis was determined from its
specific activity ($\mu Ci/\mu g$).

15   The invention will now be illustrated by reference
to the following non-limiting examples.

Example 1   Antiserum Characterization

Antisera against 58kD and 31kD inhibin were character-
ized by showing that following immunization, parallel changes in
plasma FSH and inhibin antibody titre were observed, indicating
inhibin neutralization in vivo. The antisera neutralized bFF,
hFF and purified 31kD and 58kD inhibin activity in an in vitro
bioassay. The results described below refer to anti-58kD
inhibin, but similar results were obtained using anti-31kD
inhibin.

(a)   Response In Vitro to Immunization

Following the first booster (Fig. 2a) the antibody titre
was assessed by the ability of the antibody to neutralize inhibin
bioactivity in vitro and its capacity to bind ^{125}I-58kD inhibin.
A sharp parallel elevation in these activities was observed
between 1-8 weeks post-booster injection. During this period
significant (P 0.05) elevation in serum FSH (6.63 ± 0.95 ng/ml,
n = 5 vs 4.97 ± 0.87 ng/ml, n = 6 (mean ± 1 SD)) was noted.
Following the second booster (Fig. 2b), an immediate and
sustained elevation of serum ^{125}I-31kD inhibin-binding capacity
and serum FSH was observed.

Basal levels of serum FSH were assessed from the mean + 2 S.D.
of 14 observations over the preceeding five months (hatched
area Figure 2b).

These results indicate that purified inhibin from
5 bovine follicular fluid can be used to immunize rabbits,
producing an antiserum which has the capability of
neutralizing inhibin bioactivity. The elevated levels of
plasma FSH in the rabbit observed during the period of peak
antiserum titre (as assessed by iodinated inhibin binding and
10 in vitro neutralizing capacity) indicate that the antibodies
produced are capable of neutralizing endogenous inhibin. The
combination of the neutralization of inhibin both in vivo and
in vitro and the close relationship of the neutralizing
activity and iodinated inhibin binding capacity of the
15 antiserum provides convincing evidence of its specificity.

(b)   Inhibin Neutralisation In Vitro

Bovine follicular fluid inhibin (2 units) was
quantitatively neutralised by 1 μl antiserum per culture well
while 75% inhibition of bioactivity was achieved with 0.35 μl
20 per well (Fig. 3, Table 1). In Figure 3, the vertical dotted
line indicates the volume of antiserum required to achieve 75%
neutralization of inhibin activity, an arbitrary parameter of
antiserum neutralizing titre. Purified 58 and 31kD inhibin
gave corresponding 75% inhibition values of 0.33 and 0.38 μl
25 respectively. In comparison, neutralisation of hFF inhibin
bioactivity required 1.32 μl antiserum (n = 2, Table 1)
corresponding to 27% cross-reactivity in comparison to bFF
inhibin. Inhibin from ovine sources (follicular fluid, rete
testis fluid) showed 8 and 6% cross-reactivity respectively.
30 This antiserum, at a maximum non-toxic level of 4 ul
per well, did not neutralise 2 units of inhibin activity in
rat ovarian cytosol extracts.

TABLE 1.

Cross-reactivity of inhibin from various sources as assessed by inhibin neutralization in vitro and radioimmunoassay

Preparation	IN VITRO NEUTRALIZATION ^{125}I-58kD Inhibin			RADIOIMMUNOASSAY ^{125}I-31kD Inhibin		^{125}I-58kD Inhibin	
	Inhibin Bioactivity U/ml	Antiserum Titre (µl)	% Cross-Reactivity	ED_{50} (U)	% Cross-Reaction	ED_{50} (U)	% Cross-Reaction
Follicular Fluid							
Bovine	5200	0.35 ± 0.04 (6)	100	1.30 ± 0.25 (9)	100	0.99 (2)	100
Human	168 ± 32 (6)	1.32 (2)	27	5.3 ± 1.6 (4)	28.4 ± 8.7 (4)	2.7 (2)	37
Ovine	27,000 (2)	4.4 (2)	8.0	>320	< 0.3	>320	< 0.1
Ovarian Extract							
Rat	384 (2)	> 6 (2)	< 6.0	**		**	
Rete Testis Fluid							
Ovine	1040*	> 6.0 (1)	< 6.0	> 52	< 2	>52	< 2

* U/mg protein

** No displacement with 16 U inhibin

    Mean ± SD (n)

Example 2   Iodination of inhibin

Iodination of either 58kD or 31kD inhibin has been
achieved using a conventional Chloramine T iodination
procedure and was associated with considerable iodination
5 damage. Purification of the tracer was therefore necessary
and it was not achieved following gel filtration
chromatography on Sephadex G25. Specific binding of either
iodinated hormone to Matrex Red A achieved satisfactory
purification although recoveries were low. Either iodinated
10 inhibin form thus purified had the physico-chemical properties
of its non-iodinated form.

Alternative iodination procedures using Iodogen,
Iodobeads, lactoperoxidase, or Bolton-Hunter reagent were
found to cause less damage to the inhibin molecule, but
15 resulted in poorer incorporation of radioactivity.
Consequently iodination using Chloramine T was preferred.

Purified 58kD or 31kD inhibin (1-2 µg in 25 µl
electroelution buffer was added to 25 ul 0.5 M phosphate
buffer, pH 7.2. $Na^{125}I$ (0.5 mCi, 5 µl; Amersham, Bucks, UK)
20 was added. Chloramine T (40 µl) was added at a ratio of 8:1
Chloramine T to hormone. The reaction proceeded for 60
seconds at room temperature with stirring and was terminated
with 20 µl sodium metabisulphite (3 mg/ml). The reaction
mixture was made up to 50 µl in 20mM phosphate buffer 0.1% BSA
25 or 0.5% Polypep (Sigma, St. Louis, Mo., USA) pH 6.0 and gel
filtered on a Sephadex G25 column (PD10, Pharmacia, Uppsala,
Sweden) to remove ^{125}I. The void volume fractions were
pooled, made up to 20 ml and applied to a column of 200 µl
Matrex Red A (Amicon, Danvers, Mass., USA) and then washed
30 with phosphate buffer containing 400 mM KCl, the eluted counts
being discarded. ^{125}I-inhibin was eluted with 1M KCl/4M urea
in phosphate buffer. The iodinated inhibin was further gel
filtered on a Sephadex G25 column (PD10) with the appropriate
RIA buffer (see below) to remove the KCl/urea.
35   Following iodination of 58kD and 31kD inhibin, 60
µCi and 25 µCi respectively were recovered in the void volume
fractions following gel chromatography on Sephadex G25.

Approximately 30% was eluted with the 1M KCl/4M urea buffer.
^{125}I-inhibin, as assessed by its molecular weight on SDS-PAGE,
was found in this fraction.

      The specific activity of the iodinated preparations
5 was assessed in the radioimmunoassay using a self-displacement
procedure (Marana et al 1979) with the hormone used for
iodination as standard. Specific activities of 50-60 μCi/μg
for 58kD inhibin and 24 μCi/μg for 31kD inhibin were obtained,
with recoveries ranging from 5-25%.

10 Example 3   Characterisation of iodinated inhibin
      The physico-chemical characteristics of ^{125}I-inhibin
were assessed using RP-HPLC and SDS-PAGE. A close
correspondence was observed between the radioactive and
bioactive profiles on RP-HPLC for both the 58kD and 31kD
15 preparations (data not shown). The molecular mass of
^{125}I-58kD inhibin following fractionation on SDS-PAGE was
similar to purified non-iodinated inhibin under both
non-reducing (58kD) and reducing (43kD and 15kD) conditions
except that a 58kD material of unknown identity was observed
20 in relatively low proportions (18%) under reducing conditions
(Fig. 1). The molecular weight markers employed were BSA
(bovine serum albumin) 67,000; OV (ovalbumin) 43,000; CA
(carbonic anhydrase) 29,000; GL (goose egg lysozyme) 20,300;
and GL (chick egg lysozyme) 14,300. The arrow, in figure 1,
25 refers to the point of sample application. Radioactivity
found in fractions beyond fraction 47 represents free iodine
in the solvent front. The purity of the inhibin used for
iodination as assessed by silver staining on SDS-PAGE suggests
that the ^{125}I-58kD material is not an iodinated contaminant.
30 In support, ^{125}I-58kD inhibin was fractionated by
microelectrofocusing procedure on the pH range 3.5-10 and 4-8
(Foulds and Robertson 1983), and 3 peaks of radioactivity with
pI values of 7.4, 6.2 and 5.2 were observed. Upon reduction
on SDS-PAGE each of these peaks showed persistence of
35 ^{125}I-58kD material. The results suggest that the presence of

the ^{125}I-58kD material is attributable to difficulties in
reduction of the iodinated hormone rather than to the
iodination of a contaminating protein.

Fractionation of the ^{125}I-31kD inhibin on SDS-PAGE
revealed molecular weights of 30,200 under non-reducing
conditions and 20,000 and 15,000 subunits following reduction;
these values are similar to those for the non-iodinated
hormone. A second antibody RIA system using either tracer
yielded a parallel displacement between purified 31kD and 58kD
inhibin.

Example 4   Radioimmunoassay Procedure

The assay buffer used was 10 mM phosphate, 0.15 M
NaCl, 0.5% BSA, pH 7.2. A delayed tracer addition, second
antibody assay system was employed. The sample and antiserum
were incubated in a volume of 300 µl for 16 hours at room
temperature following which ^{125}I-inhibin (10,000 cpm, 100 µl)
was added and the incubation continued either overnight at
room temperature or for 48 hours at 4°C. Second antibody
(goat antiserum to rabbit IgG, 100 µl) was added and incubated
for 1 hour at 4°C following which 1 ml 6% polyethylene glycol
was added. The tubes were vortexed and incubated for a
further 30 min, spun at 2000 g for 30 min at 4°C, decanted and
counted. The inclusion of Triton X-100 (final concentration
0.025%) in the assay buffer reduced non-specific binding from
4 to 0.5%.

Radioimmunoassay procedures were established using
both 31 and 58kD inhibin tracers. Following a logit-log dose
transformation of the response curves, linear displacement of
each tracer was observed for a range of inhibin preparations,
with the exception of 31kD inhibin when using ^{125}I-58kD
inhibin as tracer, in which a deviation from linearity below
logit -0.5 (38% B/Bo) was seen (Fig. 4). In figure 4, each
value represents the mean ± SD of triplicates. The
characteristics of each assay are outlined in Table 2.
Scatchard analysis revealed similar affinities for the
antiserum of either inhibin form. Non-parallel dose response

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lines were observed between bFF and either 31kD inhibin with ^{125}I-31kD inhibin as tracer of 58kD inhibin with ^{125}I-58kD inhibin as inhibin tracer. The sensitivity (ED_{10}) and ED_{50} values were comparable in each assay with either hormone.

TABLE 2

Characteristics of the two radioimmunoassay systems with ^{125}I-31kD inhibin and ^{125}I-58kD inhibin as tracers

	^{125}I-31kD inhibin	^{125}I-58kD inhibin
Antibody Dilution	1:8000	1:4000
Tracer binding (Bo)	30%	18%
Affinity (Kdis) x 10^{-10} M 20°C	0.66	0.72
ED_{10} (ng, fm)		
31kD inhibin	0.10, 3.0	0.13, 4.4
58kD inhibin	0.07, 1.2	0.13, 2.2
ED_{50} (ng)		
31kD inhibin	0.30, 10.1	0.51, 17.1
58kD inhibin	0.26, 4.3	0.43, 7.0
Slope*		
bFF	1.37 ± 0.09[a] (8)	1.47 ± 0.09[c] (8)
31kD inhibin	1.53 ± 0.09[b] (5)	1.68 ± 0.08 (3)
58kD inhibin	1.50 ± 0.07 (3)	1.73 ± 0.14[d] (5)
Precision**	0.036 (5)	0.038 (5)
Between Assay Variation**	14 (5)	8.5% (5)
Bio/Imm Ratio		
31kD inhibin	0.34 ± 0.09 (16)	0.43 ± 0.13 (4)
58kD inhibin	0.30 ± 0.12 (7)	0.37 ± 0.12 (5)
BGCM	0.25 (1)	–

a vs b $P < 0.05$)

)   as assessed by paired t-test

a vs c $P < 0.01$)

Mean ± SD

*   Number in brackets: number of preparations

** Number in brackets: number of assays

BGCM = bovine granulosa cell culture medium

For details see text.

Example 5   Specificity of the assay
          The specificity was assessed on the following
grounds. First, a similar hierarchy of cross-reaction of
inhibin from various species in the RIA using either tracer
5 and in vitro neutralisation studies was observed (Table 1).
The cross-reaction in the radioimmunoassay of inhibin from
different species when expressed in terms of their bioactivity
was bFF 100%, hFF 30% ovine FF 1% and rat ovarian extract
non-detectable. With respect to this antiserum it is apparent
10 that both male and female bovine and human inhibin share
common antigenic determinants not found in inhibin from the
other two species. This implies close structural similarity
between inhibin from both sexes and species. Secondly, no
cross-reaction (0.5%) occurred for a range of purified
15 glycoproteins and polypeptides. Rat LH and FSH, ovine LH and
FSH, hCG, bovine TSH, LHRH, ovalbumin and bovine serum albumin
showed less than 0.5% cross reactivity using either tracer.
Alternatively, medium from the bovine granulosa cell culture
(Fig. 4), and bovine testis extract (data not shown), both
20 containing inhibin bioactivity, gave parallel displacement
curves to bFF inhibin in the RIA. The parallel dilution of
inhibin bio- and immunoactivity of medium from bovine
granulosa cell culture with the inhibin standard provides
evidence for these cells being the site of inhibin production,
25 as has been previously suggested (Erickson and Hsueh 1978;
Henderson and Franchimont 1981). Thirdly, similarities were
observed in the profiles of both biological and immunological
activities following fractionation of bFF on gel filtration
chromatography and RP-HPLC. However, in the 40-60kD molecular
30 mass region of the Sephacryl S200 column (Figure 5a), an 8-40
fold excess of immunoactivity over bioactivity was present,
accounting for 12-18% of the recovered immunoactivity.
          A large variation in the ratio of
biological/immunological activities with charcoal-treated bFF
35 as standard was observed following fractionation of bFF
inhibin on gel filtration and RP-HPLC (Fig. 5) and between
purified inhibin preparations (Table 2). The ratios ranged

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from 0.02-2.09 in fractions obtained during the purification
procedure and from 0.30-0.43 with the purified inhibin
preparations.

It is concluded that the RIA procedures are not
detecting molecular entities devoid of biological activity and
vice versa except in the lower molecular weight region
(40-60kD) of the Sephacryl S200 chromatogram. Whether this
lower molecular weight material represents a protein distinct
from inhibin which cross-reacts in the RIA or inhibin devoid
of biological activity has not been established.

The cross-reactivities of inhibin-related proteins
in the RIA relative to 31 kDa bFF inhibin were as follows:
porcine transforming growth factor-beta (R & D Systems, Minn.
USA) <0.9%, bovine Mullerian inhibitory substance (kindly
provided by Dr. J. Hutson, Royal Children's Hospital,
Melbourne) <0.3% purified bovine inhibin B subunit dimer <2%
and the subunits of 31 kDa bFF inhibin obtained following
reduction and alkylation <0.1%. A range of glycoproteins and
growth factors have been previously tested (McLachlan et al,
1986) and showed cross reactivities against anti-58kD inhibin
of less than 1.0%. The specificity of anti-31kD inhibin was
similar, with cross-reactivities of less than 1.0%.

Example 6   Application of the Radioimmunoassay to Serum

The RIA in its application to serum required
substantial modification. Firstly 100mM phosphate buffer pH
7.4 containing 0.15M NaCl, 0.5% BSA was used, and, because of
its stability in serum, ^{125}I-31kD inhibin was preferred to
^{125}I-58kD inhibin as RIA tracer. Secondly, a
temperature-dependent interference of steer serum with
^{125}I-31kD inhibin binding to the antiserum was observed, with
an increase in binding (B/Bo) from 57% at 4°C to 94% at 37°C
(Fig. 7). This figure demonstrates the temperature dependence
following a 16 hour incubation in the presence of various
serum and inhibin preparations (bFF, 31kD inhibin, steer serum
(SS), cow serum (CS), bull serum (BS), human post-menopausal
serum (PMS), human female serum pool (NFP)). Tracer binding
(B/T) was maximal at 30°C in the presence of steer serum,
being 87.1 ± 3.4% (n = 5). Displacement of ^{125}I-31kD inhibin

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by bFF or 31kD inhibin was largely unaffected by temperature
Human PMS showed no temperature related interference upon
binding although the binding was elevated (B/Bo 110-120%).

5   Based on these data, conditions for the assay of
either bovine or human serum inhibin were established. These
involved using ^{125}I-31kD inhibin as tracer in an overnight
30°C incubation and, in order to compensate for the low level
of interference by SS or PMS (presumed to contain no inhibin)
standards and samples were diluted in SS or PMS accordingly.

10   No detectable activity was determined in steer or in
human post-menopausal serum, whilst bull and human female
serum showed parallel dose-response curves to their respective
follicular fluid standards, with circulating levels of 0.9 and
1.1 ng respectively.

15   Inhibin preparations or serum samples were diluted
in SS or PMS to a sample volume of 200 µl. Antiserum (100 µl,
final dilution 1:8000) and samples (200 µl) were incubated for
4 hours at 30°C, followed by a further incubation of 16 hours
at 30°C in the presence of tracer. Second antibody was added
20 and the tubes were incubated for 24 hours at 4°C, following
which 2 ml 0.15M NaCl was added and the tubes were
centrifuged.

Thirdly, with respect to a choice of standard in the
RIA of bovine serum inhibin, purified 31kD inhibin is favoured
25 in view of its stability in serum. In the absence of a
purified human inhibin preparation, 31kD bovine inhibin may be
used as the standard in the RIA of human serum inhibin.
However, the partially purified hFF inhibin preparation
described above is preferred, and purified hFF inhibin, when
30 available, would be the most preferred standard. The
detectable levels of inhibin immunoactivity in serum from
women under going ovarian stimulation with exogenous
gonadotrophin is analogous to the findings of Lee et al
(1982), where circulating levels of inhibin activity were
35 detected in PMSG treated immature female rats, particularly
directed against 58kD and 31kD inhibin.

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Individual antisera may behave differently in the assay, and assay parameters may have to be determined for each case. Considerable variations in sensitivity between antisera have been observed, particularly between antisera directed against 31kD and 58kD inhibin. Anti-31kD inhibin appeared to give greater sensitivity than anti-58kD inhibin in the samples tested so far.

Example 7   Improved Sensitivity RIA for human serum

            In order to improve assay sensitivity, the assay
procedure above was modified as follows: the total volume of
the assay was reduced from 400 to 300 μl (comprising 200 μl
5 sample, 50 μl tracer and 50 μl antiserum). The assay buffer
was 150 mM phosphate, 0.2% BSA pH 7.4, and the incubation of
sample and antiserum was 4 days at 4°C followed by the
addition of tracer and a further 3 days at 4°C prior to the
addition of second antibody. Using this method, a 2.5 fold
10 increase in sensitivity was achieved. This modified assay
procedure has been applied to the measurement of human plasma
inhibin. The modified assay allows the quantification of
plasma inhibin in normal male plasma and in plasma throughout
the normal menstrual cycle.

15 Example 8   Stability of ^{125}I-31kD and ^{125}I-58kD Inhibin in
            Serum

            SDS-PAGE profiles of ^{125}I-58kD inhibin following
overnight incubation with serum (SS and PMS) showed an
increased formation of an ^{125}I-30kD component (12% of
20 recovered activity at 4°C; 17% at 30°C) in comparison with
either buffer or bFF (6% 4°C and 30°C; (Fig. 6). The tracers
were incubated overnight at either 4 or 30°C with either bFF,
steer serum (SS) or human post-menopausal serum (PMS).
Incubation of either tracer in RIA buffer alone gave similar
25 profiles to the bFF incubation shown. Molecular weight
markers are described in Figure 1. No radioactivity was
observed between the position of the marker carbonic anhydrase
and the solvent front with either tracer. Results are
presented as the mean ± SD of three replicate experiments. In
30 contrast, SDS-PAGE profiles of ^{125}I-31kD inhibin under the
same incubation conditions showed no significant changes.
Recoveries of radioactivity with either tracer were not
affected by either temperature or by the presence of bFF or
serum.

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Example 9   Radioimmunoassay of inhibin in bovine serum

      The application of the inhibin   RIA to serum from
cattle resulted in parallel logit-log dose response lines of
BS with either bFF or 31kD inhibin as standards (Fig. 8). The
5 response shown in figure 8 is for bovine and human serum,
diluted in steer serum or post-menopausal serum respectively,
in the plasma RIA system employing ^{125}I31kD inhibin as tracer.
Potential RIA standards (bFF, 31kD inhibin, hFF) were assayed
in the presence of either phosphate buffer (200 µl, o) or
10 steer serum (200µl, ●) or post-menopausal serum (200µl, ▲) and
their logit plots were calculated using their respective
non-specific binding and Bo values. Cow serum shows a minimal
detectable immunological response. When the immunoactivity
was expressed in terms of 31kD inhibin standard the level of
15 inhibin in BS was 0.91 ± 0.27 (n = 3) mg/ml and CS 0.1 ng/ml.
A parallel response line of HFF was observed in the RIA with
31kD inhibin and hFF as standards corresponding to levels of
1.05 = 0.07 (n = 3) ng/ml. The levels in normal plasma (n =
8) were equal to or less than the sensitivity of the assay
20 (0.1 ng/ml).

Example 10   Radioimmunoassay of inhibin in infertile human
        subjects

      The radioimmunoassay previously described was
applied to plasma and serum from post-menopausal subjects
25 (presumed to be inhibin free, n = 8) and young women with
ovarian failure (premature ovarian failure n = 2, Turner's
syndrome n = 1, ovariectomized n = 1). There was no
difference in ^{125}I-31kD inhibin binding in the assay between
these two groups and therefore post-menopausal serum was used
30 as a diluent in the assay.

Example 11   Radioimmunoassay of inhibin in human serum

      The method was applied to:

      (a)   Plasma from normal women during spontaneous
menstrual cycles from the early follicular phase until the
35 time of ovulation (n = 2, example 'FL' #27, Fig. 9). Inhibin

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immunoactivity was below the limit of assay detection prior to
day 13 and its increase correlated with the increase in
circulating levels of oestradiol (E_2), LH and FSH on day 13
and 14.

5   (b)   Inhibin immunoactivity in serum from normal
men (n = 7) was at the lower limit of assay detection.

           (c)   Plasma was obtained from 26 unselected women
undergoing their fourth cycle of ovulation induction in the in
vitro fertilization programme at the Epworth Hospital,
10 Richmond, Victoria. Briefly this involved the administration
of clomiphene citrate 100-150 mg daily on day 5-9 of the
menstrual cycle, followed by human menopausal gonadotrophin
(hMG) 75-225U daily for the next 5-7 days. Adequate
follicular development was assessed by the progressive
15 increase in plasma oestradiol and by ovarian ultrasound.
Ovulation occurred spontaneously if an endogenous LH spike was
observed, or in the absence of the latter, ovulation was
induced by administration of 5000 IU of human chorionic
gonadotrophin (hCG). For an example of a spontaneous
20 ovulation see 'BU' # 11, Fig. 9k and for an hCG-induced
ovulation sample see 'JO' 1, Fig. 9a.

           Inhibin immunoactivity in the plasma samples, as
defined in terms of the biological activity of a purified 31kD
inhibin standard, showed a highly significant correlation with
25 plasma oestradiol levels (Fig. 10). The correlation
coefficient values have been calculated from the total data in
figure 9. An example of the correspondence of plasma
oestradiol and inhibin during an ovulation induction cycle is
seen in example 'BE' # 9, Fig. 9b. There was also significant
30 correlation between peak plasma oestradiol concentrations and
the number of oocytes recovered, and peak plasma inhibin
concentrations and the number of oocytes recovered (Fig. 11),
as well as a strong correlation between the peak plasma
inhibin concentration and the number of ovarian follicles
35 detected ultrasonically prior to oocyte aspiration (Fig. 12).
It is therefore apparent that both plasma inhibin and plasma

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oestradiol are parameters of follicular development and
health, and in the majority of cases these show a close
correspondence.

   In certain examples ('BY' # 6, 'BR' # 10, Fig. 9h
and j), a dissociation between plasma inhibin and plasma
oestradiol concentrations was observed, suggesting different
regulation of these two parameters of follicular development.
As inhibin is a peptide produced by ovarian granulosa cells
and plasma oestradiol in the human is predominantly an ovarian
theca cell product, the assay of plasma inhibin is the first
direct plasma parameter of granulosa cell/oocyte health and
maturation. The dissociation of plasma inhibin and E_2 may
therefore be of therapeutic importance in that the plasma
inhibin is a direct measure of follicular development, and its
assessment may affect the timing of ovulation induction and
oocyte collection.

Example 12   hFF inhibin as standard for radioimmunoassay
   Human follicular fluid (hFF) obtained at oocyte
collection in the IVF programme was prepared for use as the
radiummunoassay (RIA) standard by two gel chromatographic
steps and reversed phase HPLC as described for bFF inhibin
(Robertson et al, 1985). This material yielded parallel dose
reponse lines to human female serum inhibin obtained from
women undergoing ovarian hyperstimulation for in vitro
fertilisation. This partially purified human follicular fluid
(hFF) inhibin standard preparation was defined in terms of its
in vitro inhibin bioactivity using an inhibin bioassay based
on the dose-related suppresion of FSH cell content (Scott et
al, 1980). This material was used as the RIA standard and
gave dose response lines parallel to serum inhibin obtained
from women undergoing ovarian hyperstimulation for in vitro
fertilization and also to that of pregnant serum.
   The unitage of the hFF inhibin standard was
calibrated in terms of an ovine testicular lymph standard
preparation of defined unitage 1 U/mg using the inhibin

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bioassay. The RIA has an interassay coefficient of variation
(CV) of 6.4% (n = 5 assay) and the sensitivity (logit + 2) was
0.37 U/ml.

5   The RIA was specific to bovine and human inhibin and
cross-reacted less than 0.3% with a range of glycoproteins and
growth factors. In addition, inhibin-related peptides
cross-reacted as follows: porcine transforming growth factor $\beta <$
0.9%, bovine Mullerian Inhibitory Substance 0.3%, purified
bovine inhibin B subunit dimer $< 1\%$ and the subunits of 31kDa
10   bFF inhibin following reduction and alkylation $< 0.1\%$. No
immunoactivity was detectable in the sera of castrate
subjects, post-menopausal women, nor in a subject with
Turner's syndrome. The RIA had an interassay coefficient of
variation of 8.3% (n = 5 assays), and a sensitivity of 0.37
15   U/ml.

Inhibin levels were determined at 1 dilution level
against the partially purified hFF standard preparation using
an iterative curve-fitting procedure (Burger et al., 1973).
In the calculation of results, a lognormal distribution of
20   individual observations (Gaddum et al; 1933) was assummed,
i.e. all calculations were performed using logarithmically
transformed values to give geometric means and 67% confidence
intervals. Statistical comparison between pregnant and
non-pregnant groups was performed using the unpaired t-test.

25   Example 13   Inhibin levels during luteal phase and early
                  pregnancy

Nineteen women presenting consecutively for
treatment in the Monash university IVF program were studied.
Clinically their infertility resulted from tubal disease (n =
30   7), endometriosis (n = 6), unkown causes (n = 5) or poor semem
quality (n = 4). The protocol of ovulation induction has been
described elsewhere (Wood and Trounson, 1984). Briefly, all
subjects received clomiphene citrate (Clomid, Merell Dow,
Sydney) 100-150 mg daily between days 5 and 9 of the cycle and
35   HMG (Pergonal, Serono, Rome) 75-225 units daily from day 6.
The dosage and duration of HMG therapy were optimized

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according to daily plasma oestradiol concentrations and
follicular size as assessed by ovarian ultrasound. HCG
(Pregnyl, Organon, Oss) 5,000 IU intramuscularly was
administered to induce ovulation, and oocyte retrieval was
5 undertaken 36 hours later. Embryo transfer was performed as
described by Wood and Trounson (1984). Blood was taken on day
1 post laparoscopy and every second day from day 2 to day 14
and sera stored for measurement of FSH, LH, β subunit hCG,
oestradiol, progesterone and inhibin. Three of the 19 women
10 became pregnant.

At various stages of gestation, a single serum
sample was obtained from each of 24 normal pregnant women.

Samples were assayed for inhibin using the hFF
inhibin standard described in Example 11. In the 16 patients
15 who did not conceive, luteal phase inhibin levels rose to a
peak level of 2.5 U/ml on day 6 and then fell to undetectable
levels by day 14. These results are shown in Figure 13.

The number of subject serum samples per day was
13-16 except at day one when only eight were available.
20 Results are expressed as the geometric mean ± 67% confidence
intervals. The broken line indicates the limit of sensitivity
of the inhibin radioimmunoassay. The number of subjects
showing non-detectable inhibin values is shown in parentheses.
Non-detectable values are not included in the mean ±
25 confidence intervals.

In three subjects who conceived, inhibin levels were
similar to non-conception cycles between days 2 and 8,
increasing thereafter and becoming significantly higher (p
0.001) than in the non-pregnant group by day 12
30 post-laparoscopy. Figure 14 shows these results, expressed as
the geometric mean ± 67% confidence intervals. The broken
line indicates the sensitivity of the inhibin
radioimmunoassay. *p<0.05, **p<0.01, ***p<0.001 comparing
hormone values for the pregnant and non-pregnant groups on the
35 same day. Significance values in the second panel refer to
serum FSH. The late luteal phase rise in serum inhibin in the

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pregnant patients coincided with both the rise in serum βhCG
and with the decline in serum FSH to values below those seen
in the non-pregnant group.

5   Serum FSH showed a significant inverse correlation
with inhibin in the luteal phase of the non-conception cycles
(r = 0.51, n = 113, p < 0.001) (Fig. 15). Similar significant
inverse relationships were observed between FSH and inhibin
when the data were analysed according to whether the
progesterone concentrations were in the normal ovulatory
10 luteal phase range (25-100) nm) or greater, (r = 0.38, n =
76, p < 0.001 vs r = 0.37, n = 37, p < 0.05, slopes not
statistically different). Significant inverse correlations
also existed between luteal phase serum FSH and
progesterone (r = 0.64, n = 113, p < 0.001) and oestradiol (r =
15 0.52, n = 114, p < 0.001).

        Plasma inhibin and progesterone concentrations were
significantly correlated in the luteal phase of cycles in
which pregnancy did not occur (r = 0.81, n = 85, p < 0.001), as
were plasma inhibin and oestradiol concentrations (r = 0.65, n
20 = 85, p < 0.001). In pregnant subjects, luteal phase inhibin
levels did not show significant correlations with either
progesterone or oestradiol. Serum LH levels (not shown) fell
sharply from day 1 (21.0 [17.0-26.1] mIU/ml) to a nadir (3.5
[1.2-9.8] mIU/ml) on day 8.

25   In a separate study of serum inhibin, levels were
determined during gestation in 24 normal pregnant women. The
mean level prior to 20 weeks gestation (1.31 (0.95-1.80)
U/ml, n = 13) was significantly lower (p < 0.02) than levels
after this time (2.02 U/ml (1.32-3.10) U/ml, n = 11).

30   Thus there is a rise in circulating inhibin
concentrations during the luteal phase of stimulated menstrual
cycles and during pregnancy.

Example 4   Inhibin Levels in the Normal Menstrual Cycle

        In a further study, serum inhibin was determined daily in 6 normal women throughout the menstrual cycle, using a radioimmunoassay employing an antiserum directed to 31kD inhibin. The normalcy of the menstrual cycle was assessed from the serum profiles of FSH, LH, progesterone, and oestradiol. The increase in sensitivity of the assay using this antiserum permitted the detection of inhibin in over 97% of samples. The results are shown in Fig. 16.

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10 Advantages and applications of the assay according to the
    invention.
    1.   The assay may be used for determining inhibin
    concentration in a wide range of biological samples, such as
    serum, plasma, urine, follicular fluid, tissue homogenates,
15 and culture fluids.
    2.   The assay may be used to monitor the purification of
    inhibin from tissue, biological fluids, or culture medium, or
    to monitor transfection studies.
    3.   Inhibin levels may be used as a marker of parameters
    of reproductive function, such as granulosa cell function,
    follicular development, number of ovarian follicles following
    ovarian hyperstimulation, and foetal well-being during early
    pregnancy, and Sertoli cell function.

              It will be clearly understood that the invention in
25 its general aspects is not limited to the specific details
    referred to hereinabove.
              The following terms referred to hereinbefore are
    trade marks: Amerlex-M, Clomid, Coat-a-Count, Marcol 5 2,
    Matrex Red A, Montanide 888, Norit A, Pergonal, Polypep,
30 Pregnyl, RIA-Quant, Sephacryl, Sephadex, Triton X-100,
    Ultrapore, and Ultra-Turrax.
              References cited herein are listed on the following
    pages.

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CLAIMS:

1.   A method of immunoassay for the estimation of
inhibin in an inhibin-containing sample which comprises the
step of using an antibody directed against inhibin.

2.   A method according to claim 1 in which the antibody
is contained in an antiserum raised by injecting an animal
with an antigen selected from the group consisting of
naturally-occurring or recombinant inhibin, or sub-units,
fragments or derivatives thereof.

3.   A method according to claim 2 in which the antigen
is selected from the group consisting of preparations containing
inhibin, purified bovine 58kD inhibin, purified bovine 31kD
inhibin, human inhibin, or human or bovine inhibin or sub-units,
fragments or derivatives thereof produced using recombinant
DNA technology.

4.   A method according to claim 1 in which the antibody
is a monoclonal antibody.

5.   A method according to claim 1 in which the antibody
is capable of neutralizing inhibin bioactivity.

6.   A method according to claim 1 which further comprises
the step of using labelled 58kD or 31kD inhibin as tracer.

7.   A method according to claim 1 in which the assay is
a radioimmunoassay, an enzyme-linked immunosorbent assay, or an
immunoassay based on fluorescence detection.

8.   A method according to claim 1 in which an assay
standard is used, said assay standard being selected from the
group consisting of naturally-occurring or recombinant inhibin,
or sub-units, fragments or derivatives thereof.

9.   A method according to claim 8 in whcih the standard
displays parallelism in the assay with the samples under test.

10.   A method according to claim 8 in which the standard
is selected from the group consisting of bovine 31kD inhibin,
partially purified human inhibin, and purified human inhibin.

11.   A radioimmunoassay for measuring inhibin in a
biological sample, comprising the steps of:
            a)   incubating sample and antiserum for 4 hours to
4 days at 4° to 30°C,
            b)   adding ^{125}I-inhibin and incubating either
overnight at room temperature, for 48 to 72 hours at 4°C, or
for 16 hours at 30°C,
            c)   Adding a second antibody and incubating for 30
min. to 24 hours at 4°C,
            d)   Separating precipitate, and
            e)   counting bound ^{125}I-labelled inhibin.

12.   A radioimmunoassay according to claim 11 in which
samples to be assayed are diluted in inhibin-free serum.

13.   A radioimmunoassay according to claim 11 in which
an assay standard is used, said standard being selected from
the group consisting of naturally-occurring or recombinant
bovine 31kD inhibin and naturally-occurring or recombinant
human inhibin.

14.   A radioimmunoassay according to claim 11 in which
incubation with ^{125}I-inhibin is at 30°C.

15.   A radioimmunoassay according to claim 11 in which
polyethylene glycol is added following incubation with the
second antibody and incubated for a further 30 minutes.

16.   A radioimmunoassay according to claim 11 in which
Triton X-100 is incorporated into samples to be assayed.

17.   A method for measuring inhibin in samples such as
follicular fluid or serum from various species (including
humans) wherein concentrations of inhibin in standards are
used to derive the concentration of inhibin in the follicular

fluid or serum by competitive binding of ^{125}I-labelled inhibin
and inhibin from test samples with bovine 58kD inhibin
antiserum, followed by precipitation and counting of bound
^{125}I-labelled inhibin.

18.   A method for preparation and purification of
^{125}I-labelled inhibin tracer which comprises the steps of
iodination of inhibin using a Chloramine T procedure and
purification of ^{125}I-inhibin by an affinity fractionation step.

19.   A method according to claim 18 in which the affinity
fractionation step uses Matrex Red A.

20.   A method according to claim 18   which additionally
comprises a gel filtration step.

21.   An assay standard for estimation of inhibin according
to the method defined in claim 1, claim 11 or claim 17, selected
from the group consisting of naturally-occurring or recombinant
inhibin, or fragments or derivatives thereof.

22.   An assay standard according to claim 21 selected
from 31kD bovine inhibin and human inhibin.

23.   A test kit for the estimation of inhibin in a
sample, comprising an agent selected from the group consisting
of:
          a)   labelled inhibin,
          b)   antibody directed against inhibin,
          c)   an assay standard as defined in claim 21.

24.   A test kit according to claim 23 in which labelled
inhibin is prepared according to the method of claim 18.

25.   A test kit according to claim 23 in which the
antibody to inhibin is contained in an antiserum as defined
in claim 2.

26.   Products and processes substantially as hereinbefore
described with reference to the accompanying drawings.

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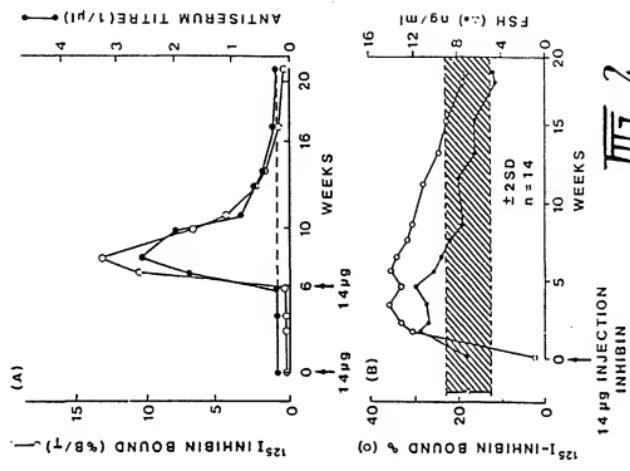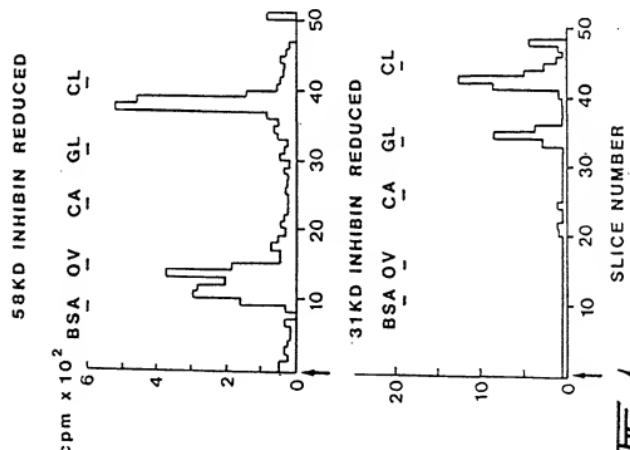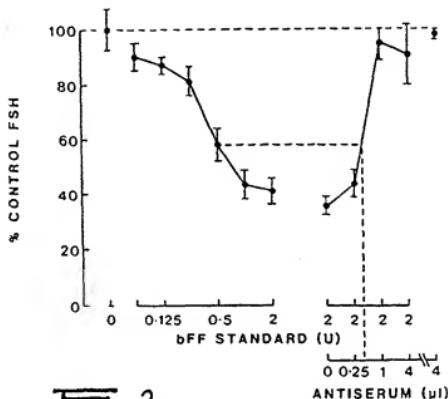
FIG. 2.

FIG. 1.

FIG. 3.

FIG. 4.

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FIG. 6.

FIG. 7.

FIG. 8.

Fig. 9 .

Fig. 9. (d) (e) (f)

FIG. 9.

FIG. 9.

Fig. 9.

FIG. 9.

FIG. 9.

Fig. 9.

FIG. 9.
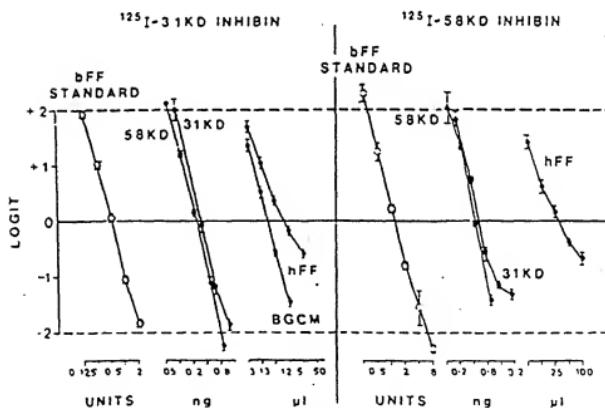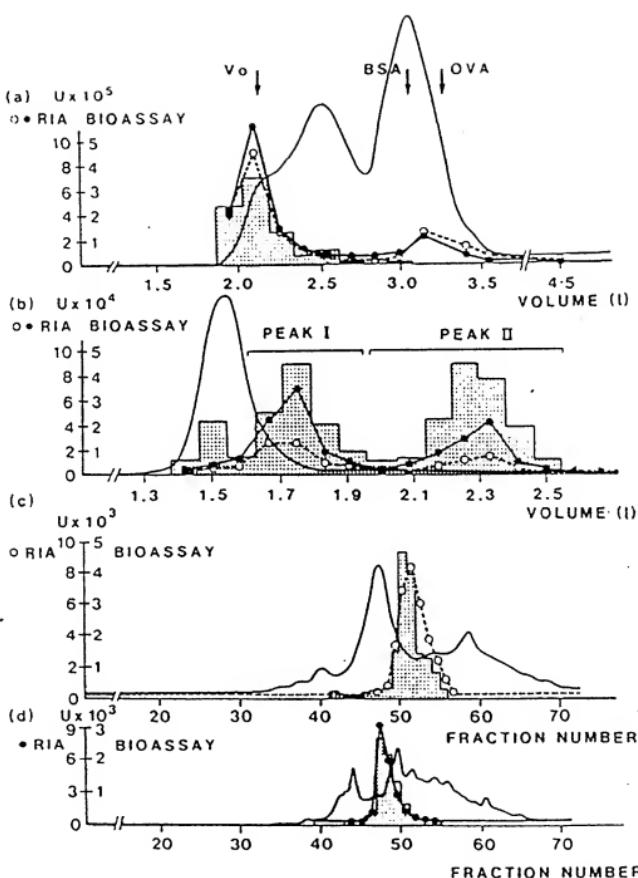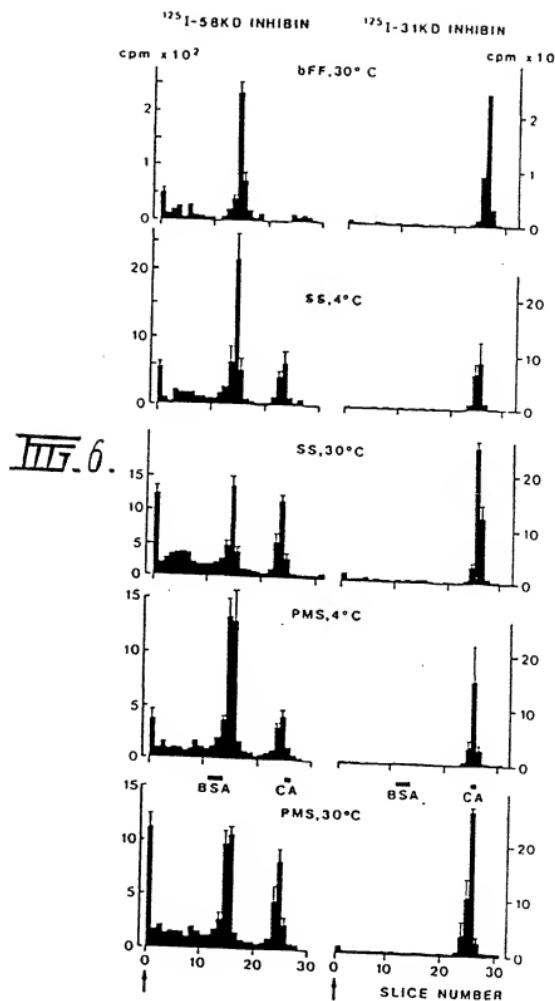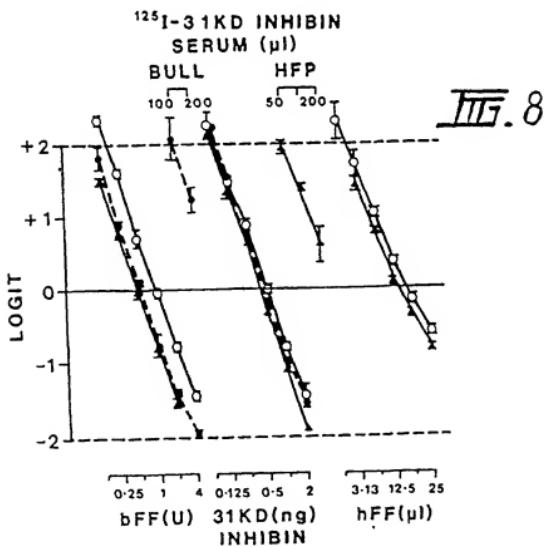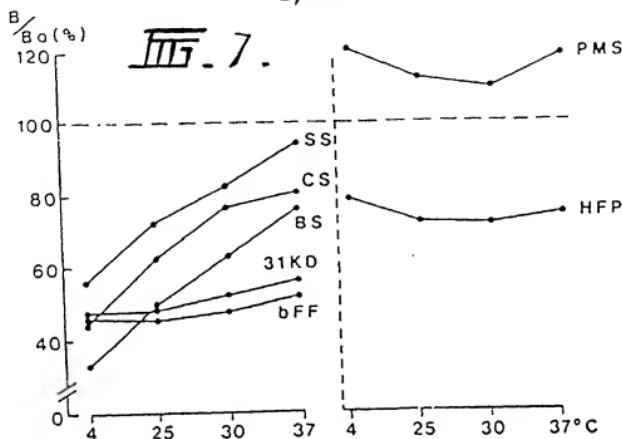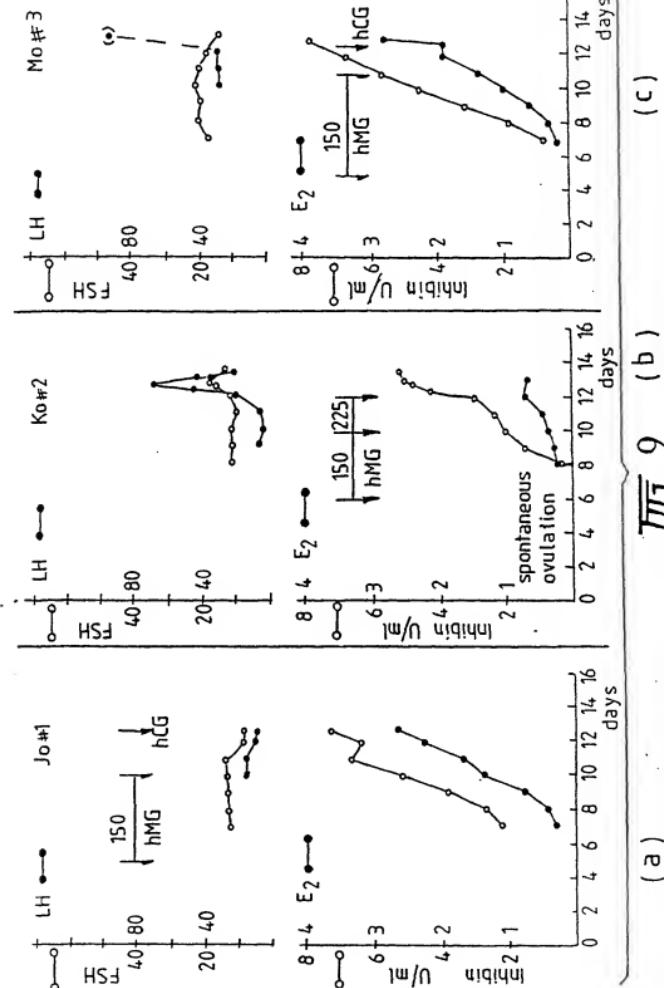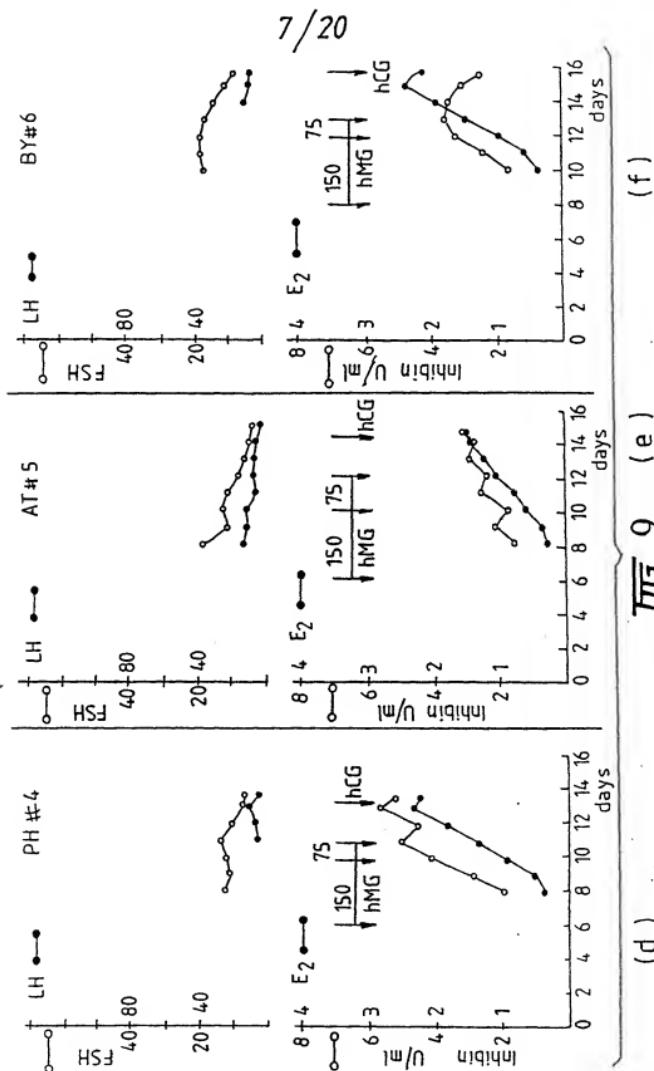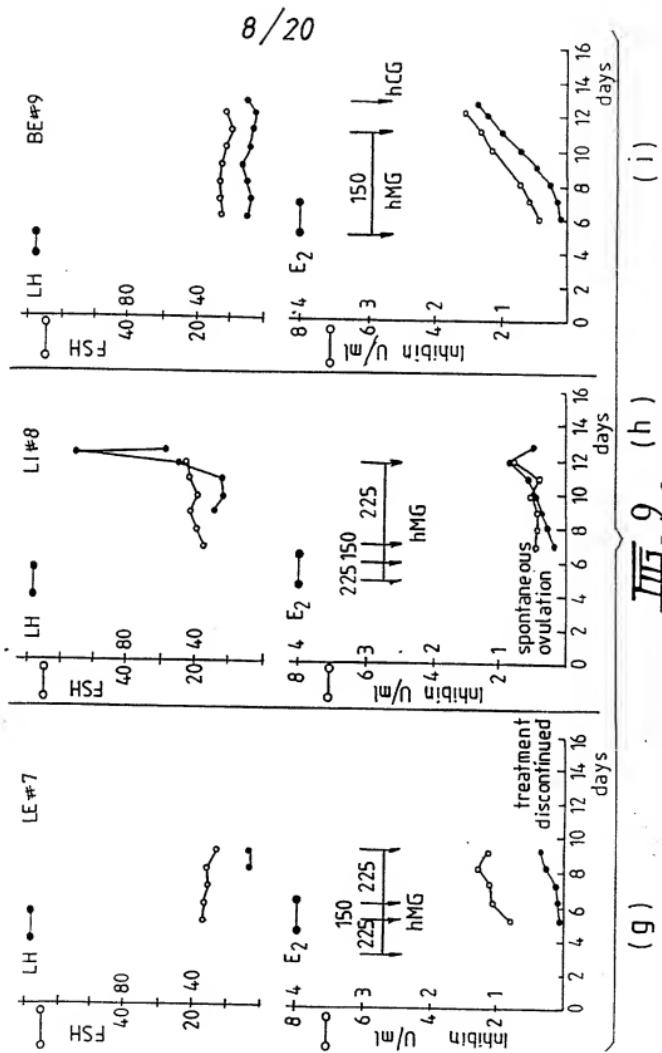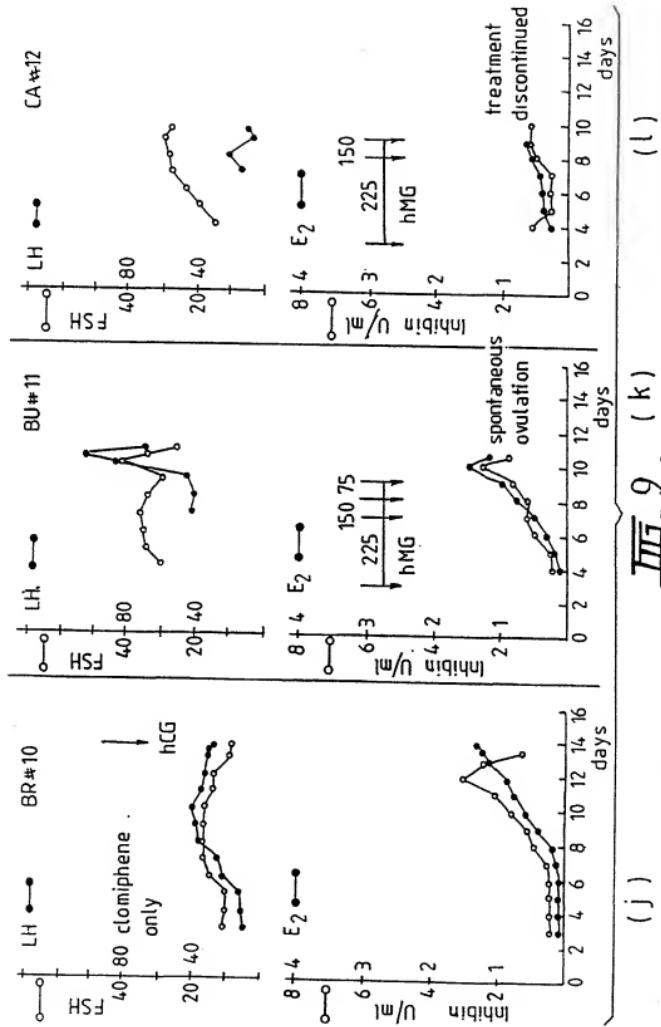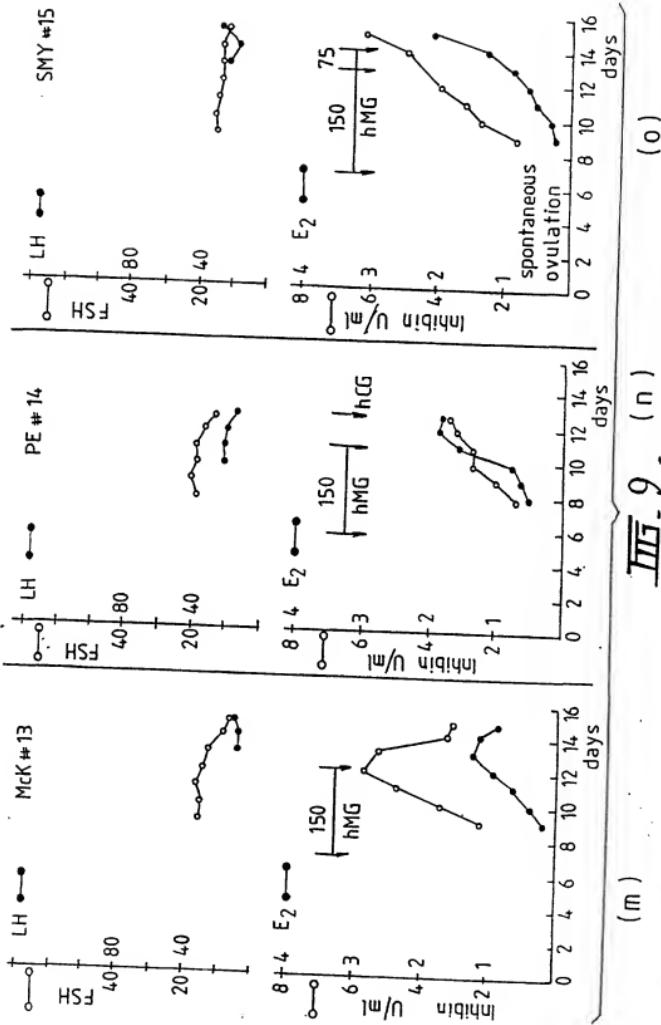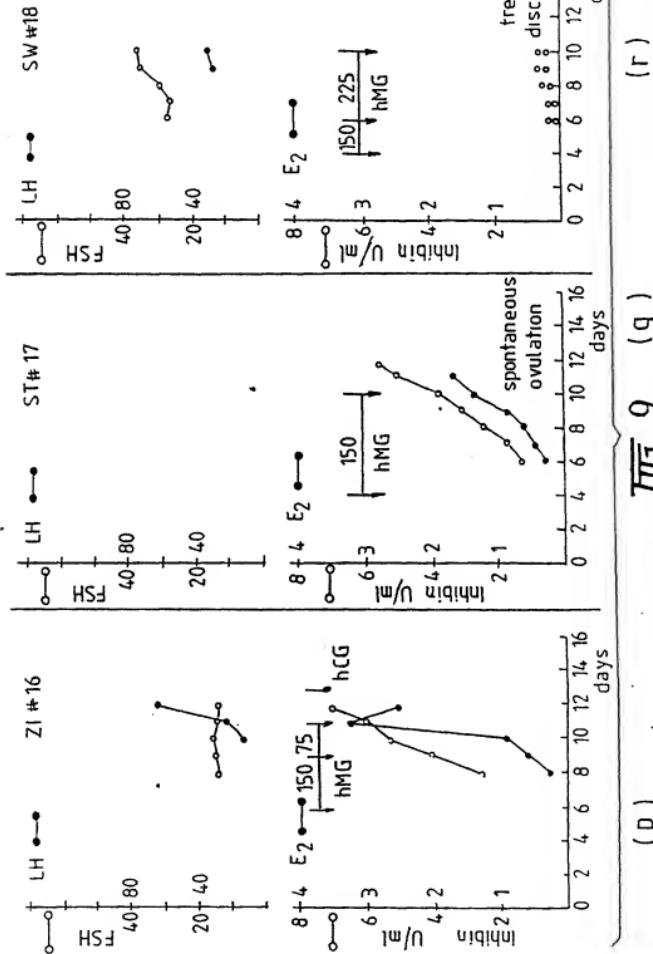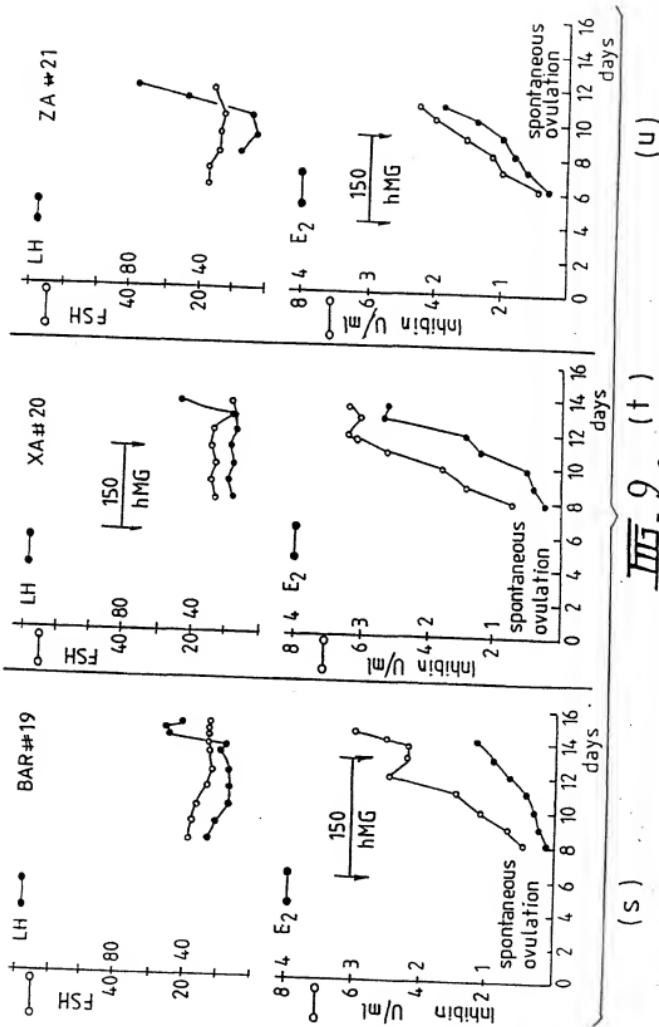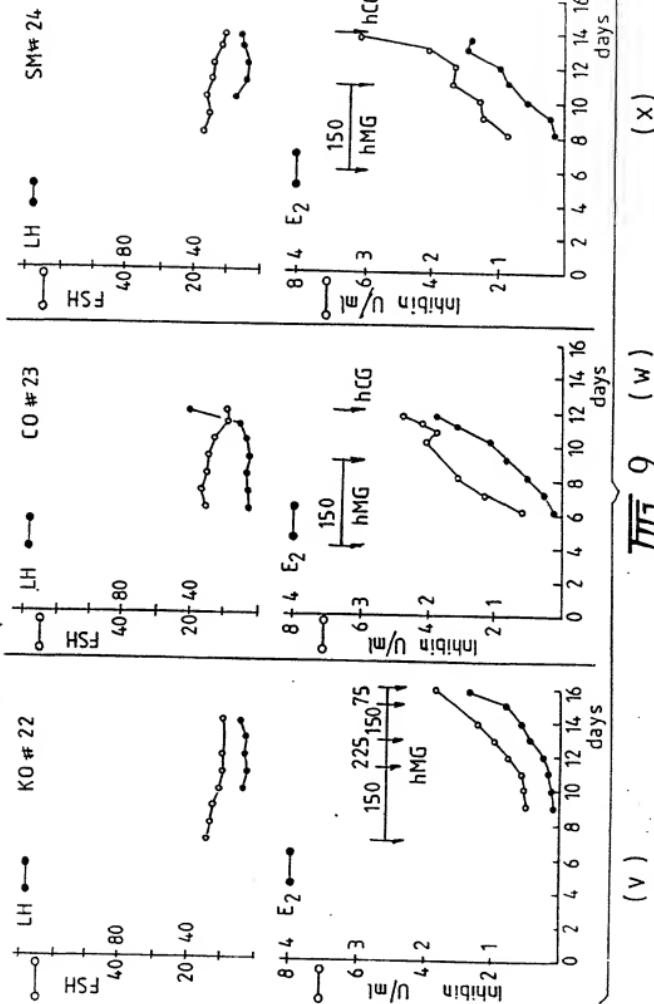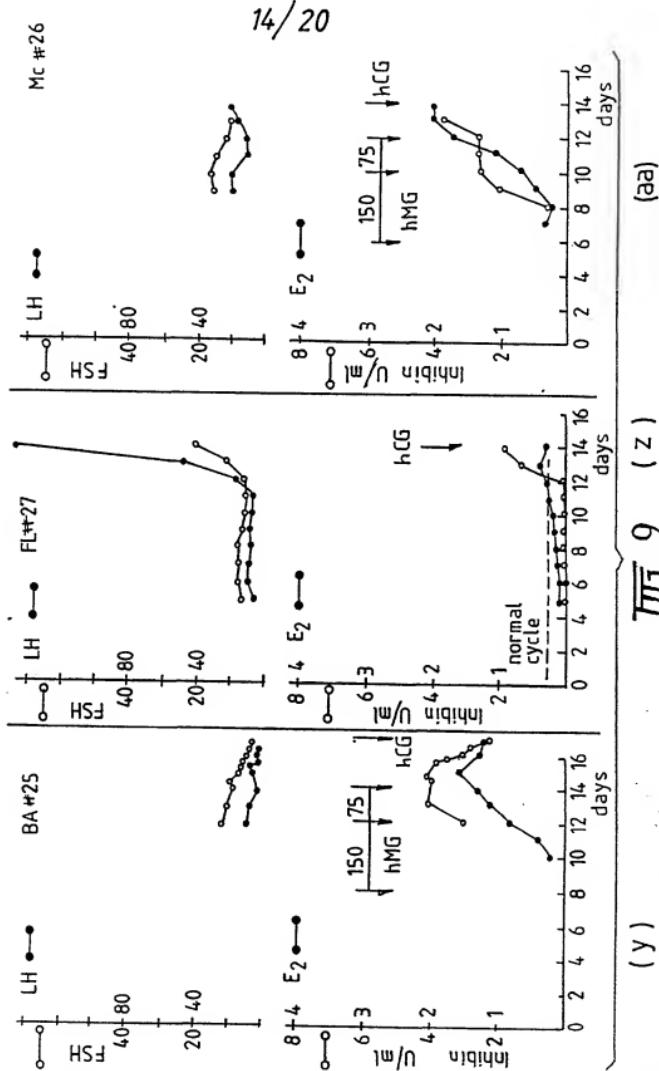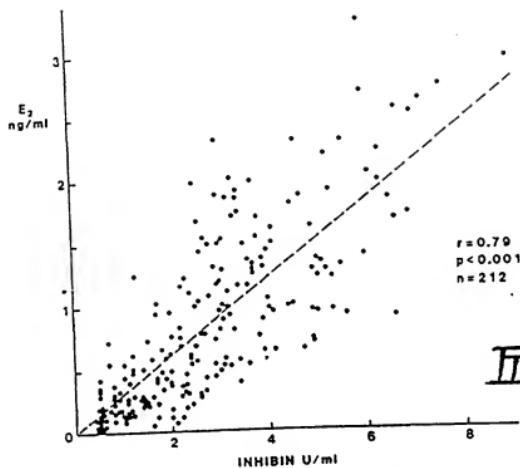
FIG. 10.
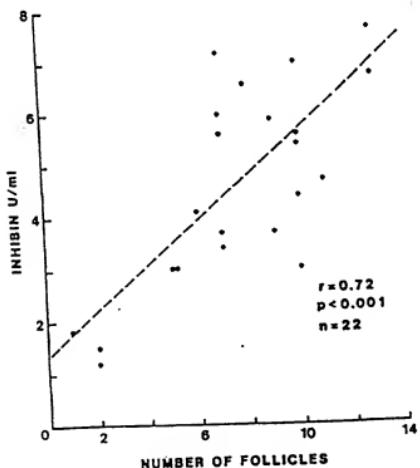
FIG. 12.
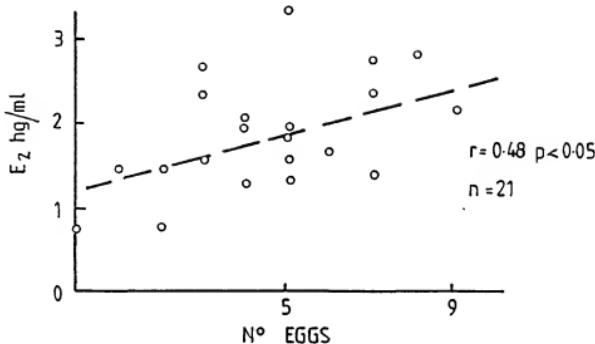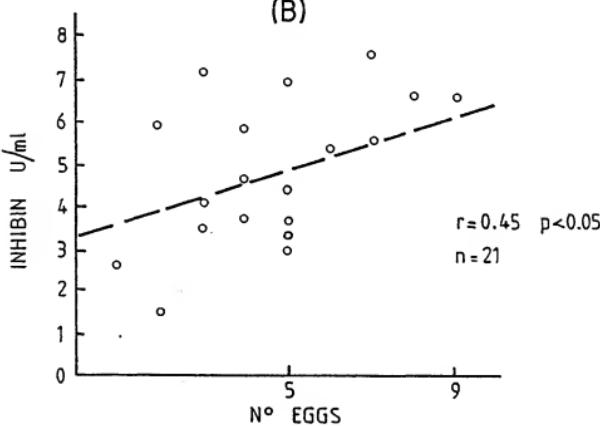
FIG. 11. Comparison of eggs recovered at Laparoscopy and Peak Plasma levels of E_2 and INHIBIN

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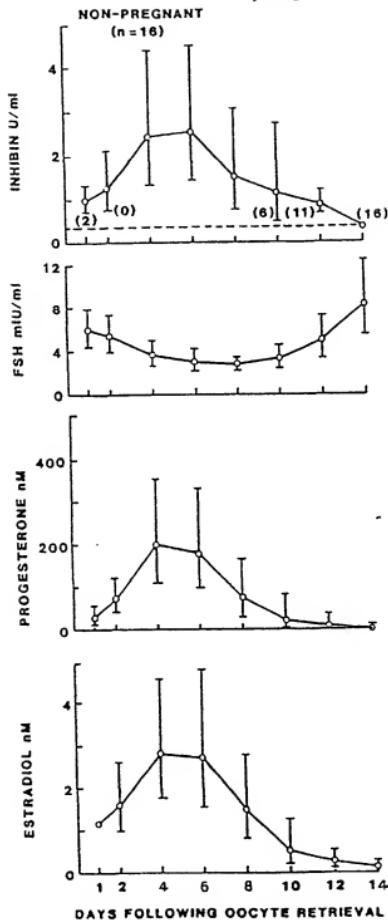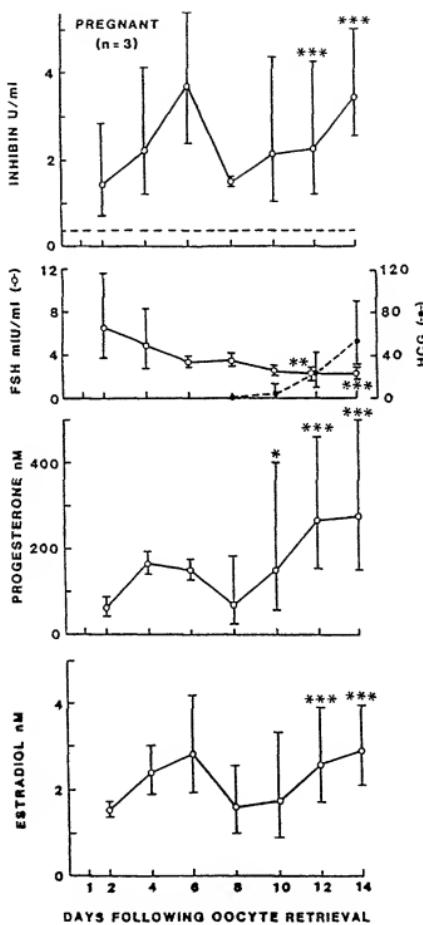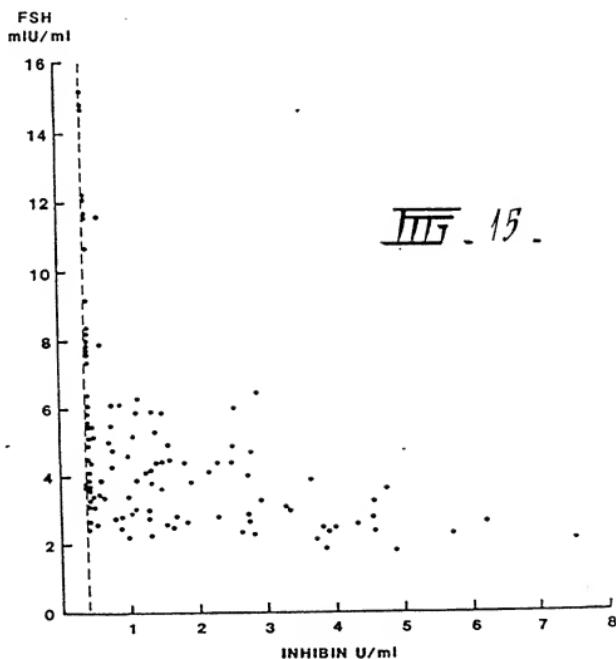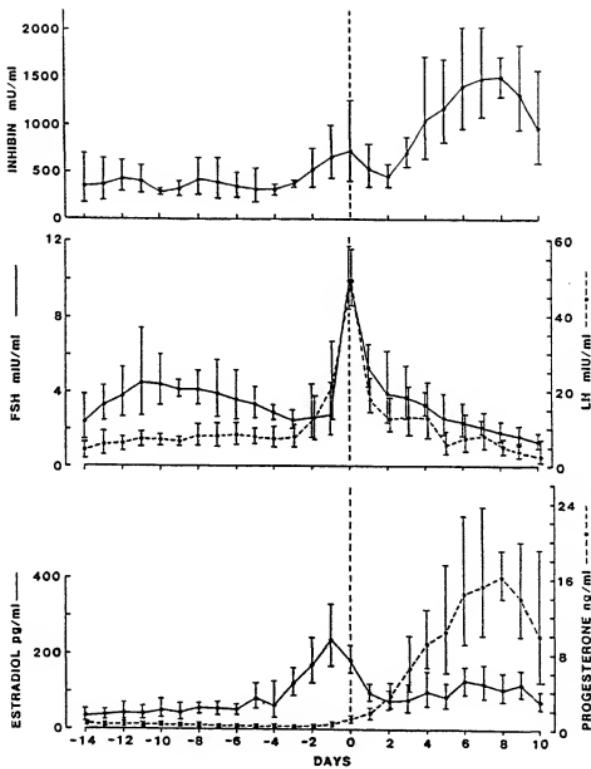
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FIG. 14.

DAYS FOLLOWING OOCYTE RETRIEVAL

FIG. 15

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Fig. 16.

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]
According to International Patent Classification (IPC) or to both National Classification and IPC
Int. Cl.[4]   G01N 33/541, 33/74, C07K 15/12

II. FIELDS SEARCHED

Minimum Documentation Searched [7]	
Classification System	Classification Symbols
IPC	Derwent World Patent Index, Keyword "INHIBIN"

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]
AU: IPC G01N 33/53, 33/577, 33/541, 33/54, 33/74, C07G 7/00, C07K 15/12

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]		
Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No [13]
X	International Journal of Gynaecology and Obstetrics, Volume 21, No.6, issued 1983 (Limerick, Ireland), S.P. Dandekar et al, "Levels of immunoreactive inhibin-like material in urine during the menstrual cycle", see pages 443-450.	ALL
X	S.M. McCann et al (Editors), Role of Peptides and Proteins in Control of Reproduction, Proceedings Workshop 1982, published 1983 by Elsevier (New York), F.H. DeJong et al, "Assay and Purification of Inhibin" see pages 257-273, especially pages 269-272.	ALL
X	Molecular and Cellular Endocrinology, Volume 44, No.3, issued 1986, March (Limerick, Ireland), D.M. Robertson et al, "Isolation of a 31 kDa form of inhibin from bovine follicular fluid", see pages 271-277, especially pages 273-276.	ALL
X,P	Molecular and Cellular Endocrinology, Volume 46, No.2, issued 1986, July (Limerick, Ireland), R.I. McLachlan et al, "The radioimmunoassay of bovine and human follicular fluid and serum inhibin", see pages 175-185.	ALL
X,P	WO,A, 86/06076 (BIOTECHNOLOGY AUSTRALIA PTY LTD et al) 23 October 1986 (23.10.86) see especially pages 47-53	ALL

* Special categories of cited documents: [10]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION	
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22 June 1987 (22.06.87)	(07.07.87) 7 JULY 1987
International Searching Authority	Signature of Authorized Officer
Australian Patent Office	J.G. HANSON

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	WO,A, 86/00078 (MONASH UNIVERSITY et al) 3 January 1986 (03.01.86)	ALL
X	Biochemical and Biophysical Research Communications, Volume 133, No.1, issued 1985, November 27 (New York, New York), J. Rivier et al, "Purification and partial characterization of inhibin from porcine follicular fluid", see pages 120-127.	1,2,5
X,P	Biochemical and Biophysical Research Communications, Volume 136, No.3, issued 1985, May 14 (New York, New York), K. Miyamoto et al, "Demonstration of high molecular weight forms of inhibin in bovine follicular fluid (bFF) by using monoclonal antibodies to bFF 32K inhibin", see pages 1103-1109.	1,3,4,7 continued

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers............, because they relate to subject matter not required to be searched by this Authority, namely:

2.☐ Claim numbers............, because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3.☐ Claim numbers............, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this International application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2)) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X,P	Journal of Endocrinology, Volume 109, No.3, issued 1986, June (Colchester, Essex, England), V. Lee et al, "Monoclonal antibody to rat ovarian inhibin", see pages 379-383.	1,4,5,7
X,P	Journal of Endocrinology, Volume 111, No.2, issued 1986, November (Colchester, Esses, England), S. Van Dijk et al, "Sexual dimorphism in immuno-neutralization of rat and ovine inhibin", see pages 255-261.	1,2,3,5
X,E	AU,A, 63512/86 (GENENTECH INC.) 9 April 1987 (09.04.87) see page 50.	1,2,7
X	Advances in Experimental Medicine and Biology, Volume 147, issued 1982 (New York, New York), F.H. De Jong et al, "Purification, characterization, and in vitro production of inhibin", see pages 37-52 especially pages 42-46.	1,2,3,5,6,7
X	Biological Research in Pregnancy and Perinatology, Volume 4, No.3, issued 1983 (Munich-Deisenhofen, West Germany), J.J. Sheth et al, "Bioimmunoreactive inhibin-like substance in human fetal gonads", see pages 110-112.	1,2,3,7,8,9
X	Journal of Biosciences, Volume 7, No.2, issued 1985, March (India), A.H. Bandivdekar et al, "Isolation of inhibin-like peptides from human placenta", see pages 175-190.	1,2,3,5
X	Chemical Abstracts, Volume 102, No.17, issued 1985, April 29 (Columbus, Ohio, USA) X. Lu et al, "Preparation of inhibin from porcine semen and its radioimmunoassay", see page 64, column 1, abstract No.143 300h, Shengzhi Yu Biyun, 1984 4(4), 27-33 (Chin)	1,2,7
X	Indian Journal of Experimental Biology, Volume 23, No.10, issued 1985, October (New Delhi, India), K.S. Hurkadli et al, "Studies on immunoneutralization of inhibin: a time course study", see pages 561-565.	1,2,3,5,7
X,P	US,A, 4624944 (LI et al) 25 November 1986 (25.11.86) see columns 7-9.	1,2,3,7

This Annex lists the known "A" publication level patent family
members relating to the patent documents cited in the above-mentioned
international search report. The Australian Patent Office is in no way liable
for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members					
AU 63512/86	EP 222491	IL 80218				
WO 8606076	AU 59039/86 IL 78519	DK 6113/86	EP 218717			
WO 8600078	AU 44374/85 IL 75412	DK 605/86 NO 860427	EP 185034 ZA 8504346			

END OF ANNEX